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An update on the phylogeny and biogeographical history of Rhipicephalus sanguineus complex

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Abstract: *Rhipicephalus sanguineus* complex is among the most studied hard tick species due to its worldwide distribution and its ability to transmit several pathogens. In this study, new local data and recent global findings were used to reevaluate the evolutionary history and phylogeny of the *R. sanguineus* complex. Seventy-nine samples of *Rhipicephalus* sp., which were collected from 32 different localities of Türkiye and one locality from Northern Cyprus, were analyzed using two mitochondrial (mt 16S rDNA, mt 12S rDNA) and one nuclear (ITS2) markers. The findings from phylogenetic trees indicate the presence of a third genetically distinct lineage of *R. sanguineus* sensu lato in addition to the tropical and temperate lineages. This particular lineage is primarily distributed in the Middle East. Only this lineage of *R. sanguineus* s.l. has been shown to occur in Türkiye. Genetic analysis confirmed distinct lineages of *Rhipicephalus turanicus* in Asia and Europe, with both lineages being found within Türkiye. Ancestral area analyses were consistent with previous findings, suggesting that the Middle East + Eastern Europe region was the origin of the many members of the complex and significantly contributed to its global distribution.

Key words: *Rhipicephalus sanguineus* complex, phylogeny, historical biogeography, Türkiye, 16S rDNA

1. Introduction

The *Rhipicephalus sanguineus* complex comprises a group of hard ticks that are distributed across almost all regions of the world, holding great importance in terms of veterinary and public health. The taxonomy and systematics of this complex have been the subject of numerous studies, primarily focusing on *Rhipicephalus sanguineus* sensu lato (Feldman-Muhsam, 1952; Pegram et al., 1987a, 1987b; Zahler et al., 1997; De Oliveira et al., 2005; Szabó et al., 2005; Nava et al., 2009, Nava et al., 2015; Nava et al., 2018). The most prominent feature of *Rhipicephalus sanguineus* s.l. is its preference for dogs as hosts, thereby being transported with them to different regions of the world (Walker et al., 2000; Szabó et al., 2005; Otranto et al., 2009; Bowman, 2011; Labruna et al., 2011; Nava et al., 2015). However, the worldwide distribution of the species has led to high genetic variability, thereby making their identification increasingly challenging. One of the primary reasons for this challenge was the absence of a holotype and, consequently, the lack of an original species description. This issue has been partially resolved by designating a neotype based on a specimen collected in France in 2018 and by identifying all life stages of the species (Nava et al., 2018).

Recent phylogenetic studies on the members of *R. sanguineus* complex across various regions of the world have provided clear evidence of genetic differences between populations. For instance, these studies have demonstrated that *R. sanguineus* s.l. comprises two well-separated genetic lineages: the temperate lineage distributed in South America and Western Europe, and the tropical lineage distributed in South America and Africa (Szabó et al., 2005; Moraes-Filho et al., 2011; Dantas-Torres et al., 2013; Latrofa et al., 2013). Recent studies have further confirmed this systematic differentiation, indicating that the tropical lineage is more closely related to samples of African *Rhipicephalus guilhoni* and European *R. turanicus*, while the temperate lineage is more distantly clustered (Dantas-Torres et al., 2013; Hekimoglu et al., 2016). More recently, the temperate lineage has been defined as *R. sanguineus* s.s. (Nava et al., 2018), whereas the tropical lineage has been revised as *Rhipicephalus linneai* (Slapeta et al., 2021). Similarly, two genetically different lineages of *R. turanicus* distributed in Southern Europe and Middle East/Asia have been reported (Bakkes et al., 2020). Given that the type locality of *R. turanicus* sensu stricto is Uzbekistan (Filippova, 1997), the Middle East/Asia lineage has been suggested as *R. turanicus* s.s. (Bakkes

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et al., 2020). New genetic data for *R. sanguineus* s.l. and *R. rossicus* were obtained from Eastern European countries (Serbia, Croatia, Romania) using COI and 16S rDNA markers (Hornok et al., 2017). This study demonstrated the significance of Eastern Europe as a region where both the temperate tropical lineages of *R. sanguineus* occur sympatrically (Hornok et al., 2017). The collection of *R. sanguineus* s.l. data from 23 different countries and analysis using 12S rDNA and 16S rDNA markers has facilitated a comprehensive global-scale phylogenetic evaluation (Zemtsova et al., 2016).

The ancestral origin of *R. sanguineus* s.l. and its subsequent distribution to the different regions of the world have been crucial issues requiring resolution. The sole study on the historical biogeography of this complex to date indicates that *R. sanguineus* sensu lato originated in Europe and subsequently colonized America (Hekimoglu et al., 2016). However, this study was conducted using only one molecular marker (mt 16S rDNA), and molecular data on closely related species, such as *Rhipicephalus rossicus* and the Asian lineage of *R. turanicus*, were not included in this work.

The primary objectives of this study were to reassess the phylogeny and biogeographical history of the *R. sanguineus* complex in light of recent available genetic data and new local data from Türkiye. Taking into account the sympatric areas in Eastern Europe (Serbia and Croatia) for *R. sanguineus* s.l. and Türkiye's geographical proximity to this region, the investigation into the existence of these lineages in Türkiye represents another important aspect of this study. Additionally, the study aims to explore the hypothesis regarding whether the tropical and temperate lineages of *R. sanguineus* s.l. have diverged into distinct species and later encountered each other in Eastern Europe, or if they have consistently coexisted in that region.

2. Material and methods

2.1. Choosing samples and morphological identification

The study was conducted using 67 specimens collected from 32 localities in Türkiye and one from Northern Cyprus between 2013 and 2022 (Figure 1). The ticks were predominantly obtained from dogs, with additional collection from domestic animals via flagging method (Table 1). Sample identification was performed using morphological identification keys under a stereomicroscope (Filippova, 1997; Walker et al., 2000; Walker et al., 2003; Estrada Pena et al., 2004; Estrada Pena et al., 2018; Nava et al., 2018; Bakkes et al., 2020).

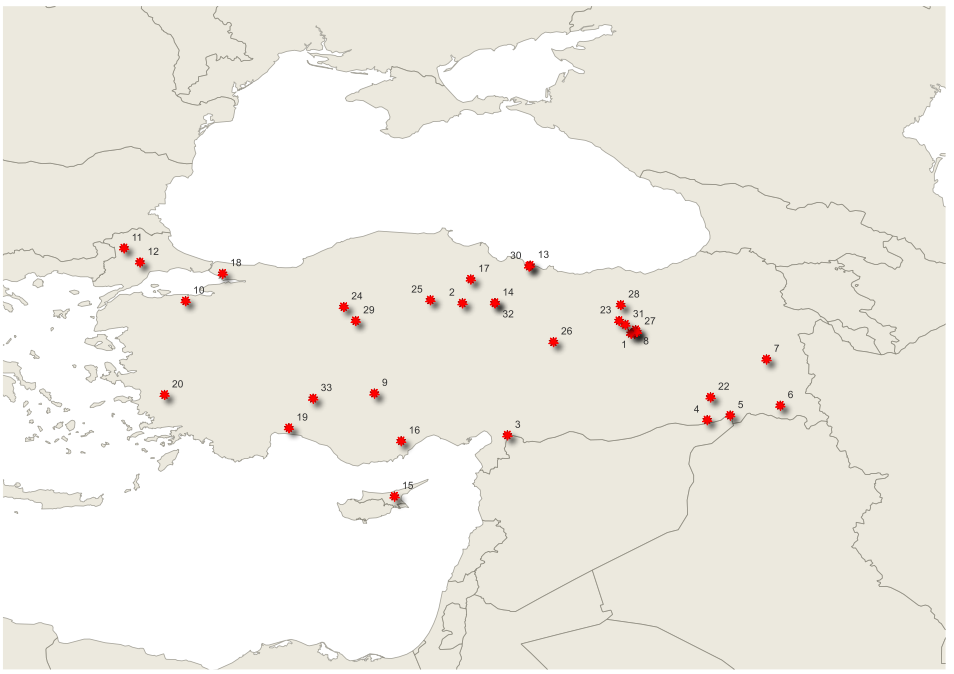

Figure 1. Map of collecting sites. (The map was generated using QGIS 3.22.2 software.)

Table 1. Coordinate information of each locality, collection methods and morphological and molecular identification of tick samples (ME): Middle Eastern lineage, (Asia): Asian lineage.

Locality	Latitude	Longitude	Sample code	Morphological identification	Molecular identification (or GenBank Accs No)	Method/ host
1. Gökçekonak/Tunceli	39.40757	39.85230	TUN20-3	*R. rossicus*	*R. rossicus*_MZ463289	Vegetation
			TUN20-2	*R. rossicus*	*R. rossicus*	Vegetation
2. Çekerek/Yozgat	40.18131	35.46488	YOZ20-29	*R. turanicus*	*R. turanicus*	Human
3. Hassa/Hatay	36.81472	36.6111	HTY1	*R. turanicus*	*R. turanicus*	Sheep
			HTY2	*R. turanicus*	*R. turanicus*	Sheep
4. İdil/Şırnak	37.200966	41.702872	GAP53-1	*R. turanicus*	*R. turanicus*	Sheep
			GAP53-2	*R. turanicus*	*R. turanicus*	Sheep
			GAP53-3	*R. turanicus*	*R. turanicus*	Sheep
			GAP52	*R. turanicus*	*R. turanicus*_MZ463284	Sheep
5. Cizre/Şırnak	37.319411	42.289588	GAP33	*R. turanicus*	*R. turanicus*_MZ463285	Sheep
			GAP58	*R. turanicus*	*R. turanicus*_MZ463286	Sheep
			GAP29	*R. turanicus*	*R. turanicus*_MZ463284	Sheep
			GAP59	*R. turanicus*	*R. turanicus*_MZ463283	Sheep
			GAP34	*R. turanicus*	*R. turanicus*_MZ463287	Sheep
			GAP27	*R. bursa*	*R. bursa*_MZ4632882	Sheep
6. Geçitli/Hakkari	37.570659	43.56591	GAP74	*R. turanicus*	*R. turanicus*_MZ463283	Sheep
7. Özyurt Village/Van	38.74944	43.21305	VAN19-3	*R. turanicus*	*R. turanicus*_MZ463294	Sheep
			VAN19-6	*R. turanicus*	*R. turanicus*	Sheep
8. Pülümür/Tunceli	39.49722	39.88000	TUN19-13(1)	*R. turanicus*	*R. turanicus*	Cow
			TUN19-13(2)	*R. turanicus*	*R. turanicus*	Cow
			TUN19-7	*R. rossicus*	*R. rossicus*_MZ463288	Cow
9. Karapınar/Konya	37.88083	33.22194	KON18-32	*R. turanicus*	*R. turanicus*	Sheep
10. Karacabey/Bursa	40.2350	28.40999	BUR18-2(1)	*R. turanicus*	*R. turanicus*	Cow
			BUR18-2(2)	*R. turanicus*	*R. turanicus*	Cow
11. Osmanlı Village/ Edirne	41.58333	26.84222	EDR18-15(1)	*R. turanicus*	*R. turanicus*	Sheep
			EDR18-15(2)	*R. turanicus*	*R. turanicus*	Sheep
12. Hayrabolu/Tekirdağ	41.22361	27.24833	TEK18-26 (2)	*R. sanguineus* s.l.	*R. sanguineus* (ME)	Dog
			TEK18-23	*R. turanicus*	*R. turanicus*	Dog
			TEK18-27	*R. turanicus*	*R. turanicus*	Dog
13. Çınarsuyu/Ordu	41.14367	37.19187	ORD18-75	*R. turanicus*	*R. turanicus*	Dog
			ORD18-76	*R. turanicus*	*R. turanicus*	Dog
			ORD71	*R. turanicus*	*R. turanicus*	Dog
14. Artova/Tokat	40.18740	36.29385	TOK17-15	*R. turanicus*	*R. turanicus*	Goat
15. Geçitkale/ Northern Cyprus	35.254	33.734	CYP5	*R. sanguineus* s.l.	R. sanguineus (ME)	Dog
			CYP7	*R. sanguineus* s.l.	*R. sanguineus* (ME)	Dog
			CYP9	*R. sanguineus* s.l.	*R. sanguineus* (ME)	Dog
16. Utalmış /Mersin	36.665	33.904	MER15-34(2)	*R. turanicus*	*R. turanicus*	Goat

Table 1. Continued

17. Suluova/Amasya	40.789	35.677	AMA15-72	*R. sanguineus* s.l.	*R. sanguineus* (ME)	Dog
			AMA15-73	*R. turanicus*	*R. turanicus* (Asia)	Dog
18. Emirli Village/ İstanbul	40.935	29.354	IST15-116	*R. sanguineus* s.l.	*R. sanguineus* (ME)	Dog
			IST15-R1	*R. turanicus*	*R. turanicus*	Vegetation
			IST15-R2	*R. turanicus*	*R. turanicus*	Vegetation
19. Pınarcık Village/ Antalya	36.997	31.043	ANT15-1	*R. turanicus*	*R. turanicus*	Dog
			ANTdog1	*R. sanguineus* s.l.	*R. sanguineus* (ME)	Dog
20. Mesutlu Village/ Aydın	37.845	27.874	AYD15-5	*R. sanguineus* s.l.	*R. sanguineus* (ME)	Dog
			AYD15-6	*R. sanguineus* s.l.	*R. sanguineus* (ME)	Dog
21. Kovuklu Village/ Tunceli	39.40666	39.76305	TUN19-18	*R. rossicus*	*R. rossicus*_MZ463290	Dog
22. Yazlıca/Siirt	37.782251	41.78804	GAP48	*R. turanicus*	*R. turanicus* (Asia)_ MZ463292	Sheep
			GAP38	*R. turanicus*	*R. turanicus* (Asia)_ MZ463291	Sheep
			GAP48-2	*R. turanicus*	*R. turanicus* (Asia)	Sheep
23. Üçkonaklar / Erzincan	39.730277	39.4575	ERZ19-1	*R. turanicus*	*R. turanicus*_MZ463295	Cow
24. Ayaş/Ankara	40.08616	32.44107	AY14-1	*R. turanicus*	*R. turanicus*	Vegetation
			AY14-3	*R. turanicus*	*R. turanicus*	Vegetation
25. Keşlik Village/ Çorum	40.26010	34.64793	COR15-11	*R. turanicus*	*R. turanicus*	Sheep
			COR15-12	*R. turanicus*	*R. turanicus*	Sheep
26. Kangal/Sivas	39.192	37.785	SIV14-9	*R. turanicus*	*R. turanicus*	Cow
27. Çobanyıldızı/Tunceli	39.448661	39.90944	TUN21-20	*R. rossicus*	*R. rossicus*	Dog
			TUN21-21	*R. rossicus*	*R. rossicus*	Dog
			TUN21-24	*R. sanguineus* complex	*R. rossicus*	Dog
28. Kaş/Gümüşhane	40.135	39.49861	GUM21-6	*R. turanicus*	*R. turanicus*	Vegetation
29. Gölbaşı/Ankara	39.730555	32.743888	ANK22-1	*R. turanicus*	*R. turanicus*	Dog
30. Kocuklu Village/ Tunceli	41.12062	37.16319	ORD-31	*R. turanicus*	*R. turanicus*	Cow
31. Değirmenköy/ Erzincan	39.6325	39.6175	ERZ21-2	*R. sanguineus* complex	*R. rossicus*	Dog
			ERZ21-3	*R. sanguineus* complex	*R. rossicus*	Dog
32. Artova/Tokat	40.187402	36.293285	TOK17-12	*R. turanicus*	*R. turanicus*	Goat
			TOK17-18	*R. turanicus*	*R. turanicus*	Goat
33. Beyşehir/Konya	37.750064	31.661536	KON17-1	*R. turanicus*	*R. turanicus*	Vegetation

2.2. DNA extraction and PCR

DNA extraction was conducted using a GeneJet Genomic DNA Purification Kit (Thermofischer Scientific) with modifications to the manufacturer's protocol. After cutting the tick from the distal portion of the idiosoma (while preserving the morphological identification features), DNA was extracted from the entire body. The remaining cuticle was preserved in 70% alcohol for further morphological examination. DNA was extracted from 64 individuals, and an additional 15 individuals, whose DNA had been isolated in previous study (Hekimoglu et al., 2021), were includedin this study (Table 1). The extracted DNA was stored at +4°C.

The PCR mixture comprised 17.5 µL of H2O, 2.5 µL of each primer (10 pmol/µL), 25 µL of High Fidelity PCR Master Mix (Thermo Scientific), and 2.5 µL of DNA. The gene regions Mt 16S rDNA and Mt 12S rDNA were amplified using primers and PCR protocols designed by Mangold et al. (1998) and Beati and Keirans (2001), respectively. The ITS2 region was amplified following the protocol outlined by Zahlet et al. (1997). Subsequently, the sequences were compared using the BLAST tool provided by the National Center for Biotechnology (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

A total of 53 PCR products for mt16S rDNA, 60 for mt12S rDNA, and 30 for ITS2 were sent for sequence analysis (Macrogen Europe).

2.3. Molecular datasets

The chromatograms were initially examined and modified using Sequencher v5.4.6 software (Gene Codes Corporation, http://www.genecodes.com). The regions containing primer sequences were also trimmed using the same program. Sequences acquired from mitochondrial markers were separately aligned for each gene region using the CLUSTAL W algorithm (Larkin et al., 2007) in MEGA11 software (Kumar et al., 2021). The resulting fasta file was analyzed using the "DNA to haplotype collapse and converter" tool of the FaBox online program (Villesen, 2007) to identify unique haplotypes and cluster sequences with identical base content. A single representative sample was chosen for each haplotype, while other samples were listed in separate column based on their similarity to this haplotype (Tables 2 and 3).

Table 2. Codes of unique haplotypes generated using mt 16S rDNA, number of individuals sharing the same base content and molecular identification results. (Previously obtained sequences from studies conducted in Türkiye are added to the list as samples sharing the same base content with the haplotypes obtained from this project. Red color indicates sequences from Hekimoglu et al., 2021, and blue color indicates sequences produced by Hekimoglu et al., 2016.)

Haplotype no	Sample code	Molecular identification	Samples sharing same base content
1	ERZ21-3	*R. rossicus*	TUN21-24, ERZ21-2, TUN20-2, MZ463290, TUN21-20, TUN21-21
2	ORD71	*R. turanicus*	ORD18-75, ORD31, KU664364
3	AMA15_73	*R. turanicus*_Asia	MZ463291
4	CYP9	*R. sanguineus*_ME	CYP5, ANT_Dog1, TEK18_26(2), CYP7, KU664365
5	AYD15-6	*R. sanguineus*_ME	AYD15-5
6	GAP53_1	*R. turanicus*	MZ463284, MZ463287
7	HTY1	*R. turanicus*	MZ463285
8	KON18-32	*R. turanicus*	HTY2, TUN13-2, AY14-3, TOK17-12, MZ463293
9	IST15-R2	*R. turanicus*	TOK17-18, VAN19-6, COR15-11, YOZ20-29, KON17-1, GUM21-6, MZ463295
10	TOK17-15	*R. turanicus*	KU664360
11	TUN13-1	*R. turanicus*	
12	EDR18-15(1)	*R. turanicus*	EDR18-15(2), KU664357
13	IST15-R1	*R. turanicus*	
14	COR15-12	*R. turanicus*	
15	ANT15-1	*R. turanicus*	
16	GAP53-2	*R. turanicus*	MER15-34(2)
17	GAP48_2	*R. turanicus*_Asia	MZ463292

Table 3. Codes of unique haplotypes generated using mt 12S rDNA, number of individuals sharing the same base content and molecular identification results.

No	Sample code	Molecular identification	Samples sharing the same base content
1	ANT-Dog1	*R.sanguineus*_ME	
2	IST15-116	*R.sanguineus*_ME	
3	HTY1	*R.turanicus*	BUR18-2(2), IST15-R1, HTY1, MER15-34(2), AY14-3, GAP52, VAN19-3, GAP58, ERZ19-1
4	IST15-R2	*R.turanicus*	
5	ANT15-1	*R.turanicus*	
6	SIV14-9	*R.turanicus*	
7	GAP53-2	*R.turanicus*	
8	GAP53-1	*R.turanicus*	
9	AY14-1	*R.turanicus*	
10	GAP33	*R.turanicus*	
11	GAP48	*R.turanicus*_Asia	
12	AMA15-72	*R.turanicus*_Asia	
13	TUN19-7	*R. rossicus*	TUN19-18
14	GAP59	*R.turanicus*	
15	*R.rossicus*_ROM	*R. rossicus*	
16	GAP27	*R.bursa*	

For the sequences acquired from the nuclear marker ITS2, chromatograms were examined to identify heterozygous nucleotide positions, and sequences exhibiting double peaks were encoded using IUPAC (The International Union of Pure and Applied Chemistry) nucleotide codes. Subsequently, these sequences were coded as two haplotypes (a and b) using DnaSP6 software (Rozas et al., 2017). The alignment and determination of unique haplotypes for this gene region were carried out similarly to the mitochondrial markers. A summary table (Table 4) was generated for the dataset produced from this gene region.

The GenBank sequences incorporated into the analysis were selected to represent various geographic regions globally and to encompass diverse lineages and species within the R. sanguineus complex. A total of 66 mt sequences for mt 16S rDNA, 44 for the mt12S rDNA, and 14 for ITS2 gene region were downloaded from GenBank for phylogenetic reconstruction. The reference and locality information for these downloaded sequences is provided in Supplementary Table 1.

As outgroups, *Rhipicephalus bursa* sequences from Türkiye (KU664348, KU664349, KU664350) for mt16S rDNA, *R. bursa* sequences from Italy (KC243833 and KC243834) for mt12S rDNA, and *R. bursa* sequence from Iran (KM986320) for ITS2 marker were included in the dataset. The dataset's characteristics for each gene region (the number of conserved, variable, and parsimony informative sites) were determined using MEGA11 software (Kumar et al., 2021). Mutations were accounted for, while indels were excluded from the final dataset analysis.

2.4. Construction of phylogenetic trees

The most suitable model for each gene region was determined using MEGA11 software (Kumar et al., 2021) based on both Bayesian and Akaike criteria (BIC, AICc). Subsequently, Bayesian Markov Chain Monte Carlo (MCMC)-based phylogenetic analyses were conducted using BEAST version 2.6.3 software (Bouckaert et al., 2019). Given that the primary objective of this study did not involve estimating the divergence time of collected ticks, the molecular clock model was set to the strict clock model with a clock rate parameter of 1. The Yule model, assuming a constant speciation rate, was chosen as the speciation model. Sampling was performed every 10,000 generations within a 100 million chain length. The XML file generated by BEAUTI v2.6.3 (Bouckaert et al., 2019) was evaluated using BEAST v2.6.3 (Bouckaert et al., 2019). A burn-in of 10% was applied to the simulations. Identical settings were employed for the analyses of the three datasets. The trees obtained and saved in (.trees file) format were consolidated into a single tree using TreeAnnotator v2.1.2 (Rambaut and Drummond, 2014) (.tre file), which was then visualized using FigTree v1.4.4 (Rambaut, 2016).

Table 4. Codes of unique haplotypes generated using ITS2, number of individuals sharing the same base content and molecular identification results.

No	Sample code	Samples sharing the same base content
1	AMA15-73	GAP53-1a, BAL17-1, GUM21-6, GAP48-2a, M185
2	CYP-9a	MER15-34(2)a, HTY1a, GAP53-2a, CYP7a, ORD31a, AYD15-5a, IST15-R1a, AYD15-6a
3	CYP-9b	MER15-34(2)b, HTY1b, GAP53-2b, ORD31b, AYD15-5b, ANTDog1b, VAN19-6b, IST15-R1b, MER15-33b, IST15-R2b, ANK22-1b, TUN13-1b, AYD15-6b
4	GAP53-1b	GAP48-2b
5	CYP-7b	
6	TUN20-2	
7	ANT-Dog1a	VAN19-6a, MER15-33a, IST15-R2a, ANK22-1a, TUN13-1a

2.5. Historical biogeography analysis

To perform an analysis of ancestral origins, new datasets were created, including 29 taxa for mt16S rDNA and 19 taxa for mt12S rDNA, each representing distinct geographic locations. Since *Rhipicephalus pumilio* was included in the ancestral area analyses in previous study (Hekimoglu et al., 2016), it was added to these datasets as well. Ancestral analyses were not conducted using the ITS2 gene region due to its inability to differentiate between different taxa within this complex.

The subdatasets created were loaded into the BEAUTI v2.6.3, and the molecular clock model was set as the strict clock model with a clock rate parameter of 1 (Bouckaert et al., 2019). The Yule model was chosen as the speciation model, and sampling was performed every 10,000 generations within a 100 million chain length. The resulting XML file was evaluated using BEAST v2.6.3 (Bouckaert et al., 2019). Subsequently, the trees obtained were consolidated into a single tree using TreeAnnotator v2.1.2 (Rambaut and Drummond, 2014).

For ancestral area analyses, the RASP 4.0 Beta (Reconstruct Ancestral State in Phylogenies) (Yu et al., 2015) was employed. This software conducts statistical dispersal-vicariance analysis (S-DIVA; Yu et al., 2010) and Bayesian binary Markov chain Monte Carlo (BBM) analyses. The S-DIVA was conducted using default parameters. In BBM, the F81 + G model was employed, and a 10 MCMC chain was run for 1 million generations with sampling occurring every 100 generations. Geographic region codes were assigned as follows: A = America, B = Western Europe, C = Africa, D = Middle East + Eastern Europe, E = Asia, and F = Australia.

3. Results

3.1. Morphological species identification

Ticks were morphologically classified as *R. turanicus*, *R. rossicus*, *R. sanguineus* sensu lato, *R. sanguineus* complex, and *R. bursa*. Among the samples, *Rhipicephalus turanicus* (74.7%) constituted the majority, while six individuals were identified as *R. rossicus* and one individual as *R. bursa*. Thirteen samples (*Rhipicephalus* sp.) could not be identified at the species level and were classified as either *R. sanguineus* s.l. or *R. sanguineus* complex (Table 1). The collection patterns based on the hosts were as follows: 36.8% from dogs, 31.7% from sheep, 10.1% from vegetation (flagging), 10.1% from cow, 10.1% from goats, and 1.2% from humans.

3.2. Molecular sequences

The Mt 16S rDNA dataset consisted of 41 sequences. Additional sequences obtained from previous studies conducted by the researchers using samples collected from Türkiye were added to the 16S rDNA dataset (Hekimoglu et al., 2016; Hekimoglu et al., 2021) (Table 1). Seventeen haplotypes were obtained using Mt 16S rDNA (Table 2). The final dataset, which included GenBank sequences from different localities around the world, consisted of 85 *Rhipicephalus* sp. sequences with a total length of 389 base pairs. Although T92 + G was determined as the model, TN93 + G was implemented instead since BEAST software does not support T92+G, and TN93+G was chosen as the closest alternative model. The total number of conserved positions in the dataset was 297, with 92 variable positions, out of which 74 were parsimony informative.

After short and unreadable mt 12S rDNA sequences were removed, phylogenetic analysis was conducted with 26 sequences and 16 unique haplotypes were obtained (Table 3). As mentioned previously, some samples were identified molecularly using mt16S rDNA (Hekimoglu et al., 2021). In this study, mt12S rDNA sequences were generated for these identified samples. The dataset, including downloaded sequences from GenBank, had a length of 342 base pairs and consisted of a total of 60 taxa. The number of conserved sites in the total dataset was 244, the number of variable sites was 98, and 75 of them were

parsimony informative. Due to the absence of T92 + G in the BEAST software, TN93 + G was employed.

Using ITS2, a total of 23 sequences were obtained, resulting in 8 unique haplotypes. This dataset comprised 22 taxa and had a length of 255 base pairs (Table 4). The best-fitting model was determined to be T92, and TN93 + G was used. The overall dataset contained 247 conserved sites and 3 variable sites, with 1 of them being parsimony informative.

3.3. Phylogenetic relationships

The phylogenetic tree constructed using mt16S rDNA identified five major clades (Figure 2): *R. sanguineus* s.s. (temperate lineage), *R. turanicus* Asian lineage, *R. turanicus* European lineage, *R. sanguineus* tropical + *R. sanguineus* Middle East lineage, and *R. rossicus*. *Rhipicephalus rossicus*, which comprised sequences from Türkiye, India, Romania, and China, is distantly located from the other clades (99%). *Rhipicephalus sanguineus* s.s. comprised samples from Europe, including France, Germany, Spain, Serbia, Croatia, as well as countries from both North (USA) and South America (Argentina, Uruguay) (100%). None of the haplotypes from Türkiye grouped within this lineage. *Rhipicephalus turanicus* Asian lineage seemed to distribute mostly in Middle Eastern countries such as Israel

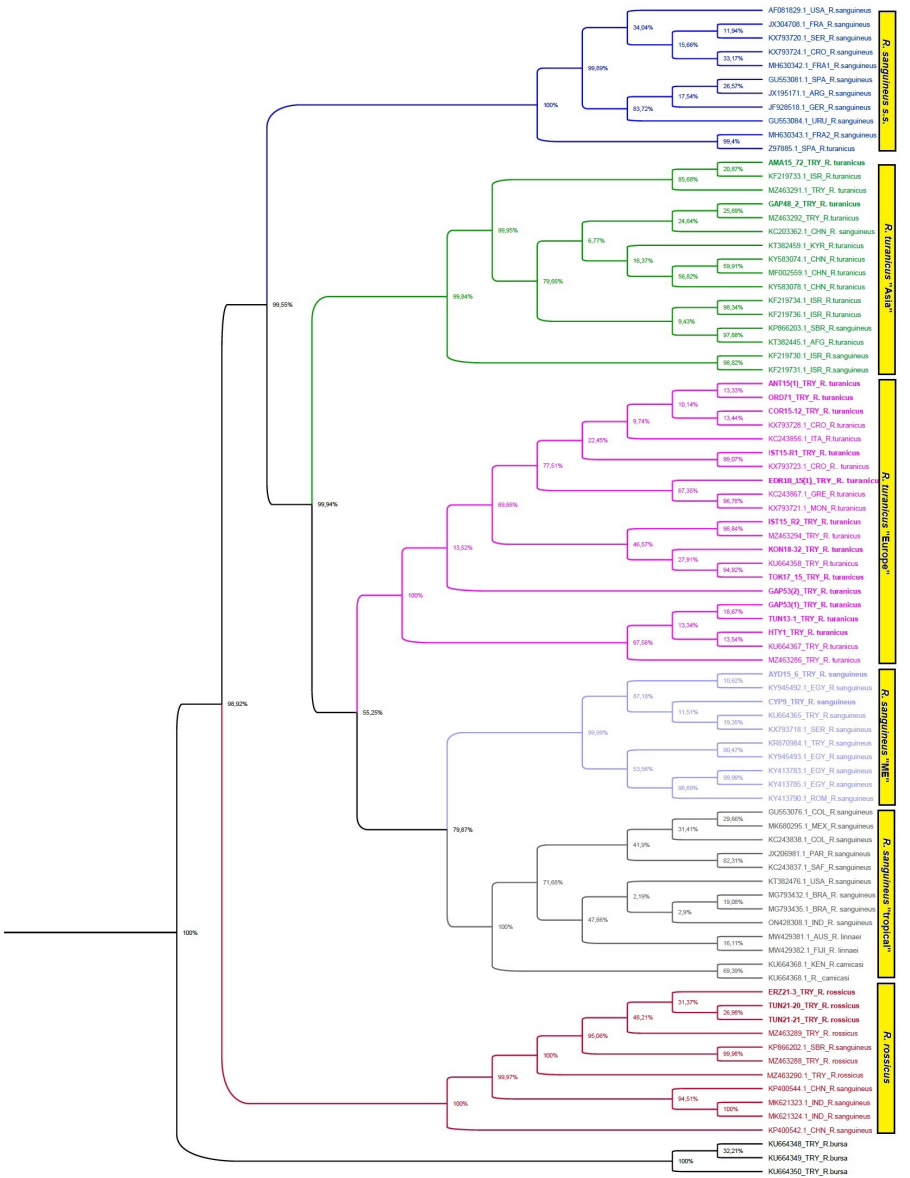

Figure 2. Phylogenetic tree of sequences obtained by mt 16S rDNA from this study and sequences of GenBank. Haplotypes obtained from this study are indicated with TRY codes and highlighted in bold.

and Türkiye, as well as countries in Asia continent such as Kyrgyzstan, China, Afghanistan, and eastern Siberia (100%). This lineage was closely related to *R. turanicus* European lineage and *R. sanguineus* tropical + Middle East lineage (55%). The majority of haplotypes from Türkiye (12/19) grouped within the *R. turanicus* European lineage and clustered together with sequences from Italy, Croatia, and Greece. The *R. sanguineus* tropical lineage is separated into two lineages (80%): One lineage involved samples of Middle East (Egypt, Romania, Northern Cyprus, Türkiye), while the other lineage included sequences from Africa, America, and Australia.

Five clades were identified in the mt 12S rDNA phylogenetic tree (Figure 3): *R. turanicus* Asian lineage, *R. sanguineus* tropical + Middle East lineage, *R. turanicus* European lineage, *R. rossicus*, and *R. sanguineus* s.s. Unlike mt 16S rDNA, *R. sanguineus* s.s. and *R. rossicus* were sister taxa (65%) according to mt 12S rDNA tree. *Rhipicephalus sanguineus* s.s. comprised sequences from America continent, such as USA, Uruguay, Argentina, as well as European countries including France and Portugal. *Rhipicephalus rossicus* consisted of sequences from Türkiye, Romania, and Russia. The majority of haplotypes from Türkiye (9/15) grouped within the *R. turanicus* European lineage, together with sequences from Greece, Switzerland, and Italy (96%). *Rhipicephalus turanicus* Asian lineage was closely related to the *R. sanguineus* tropical + Middle East lineage (77%). The *R. turanicus* Asian lineage comprised samples from Amasya and Siirt provinces in Türkiye, as well as sequences from Israel, Afghanistan, Kyrgyzstan, and Uzbekistan. *R. sanguineus* tropical lineage and Middle East lineage were sister taxa (98%). Additionally, two separate lineages were identified in Middle East lineage (100%). Sequences from Romania, Italy, and Türkiye (from Antalya and İstanbul) clustered together, while samples from Egypt and Israel formed a distinct lineage. The *R. sanguineus* tropical lineage comprised sequences from Australia, America, and Europe.

In contrast to the phylogenetic pattern observed from mitochondrial DNA sequences, ITS2 marker was unable to distinguish species within *R. sanguineus* complex (Figure 4). In the phylogenetic tree, only *R. rossicus* was identified as a distinct lineage, whereas other taxa (*R. sanguineus* s.s. and tropical lineage, and even *R. turanicus*) could not be

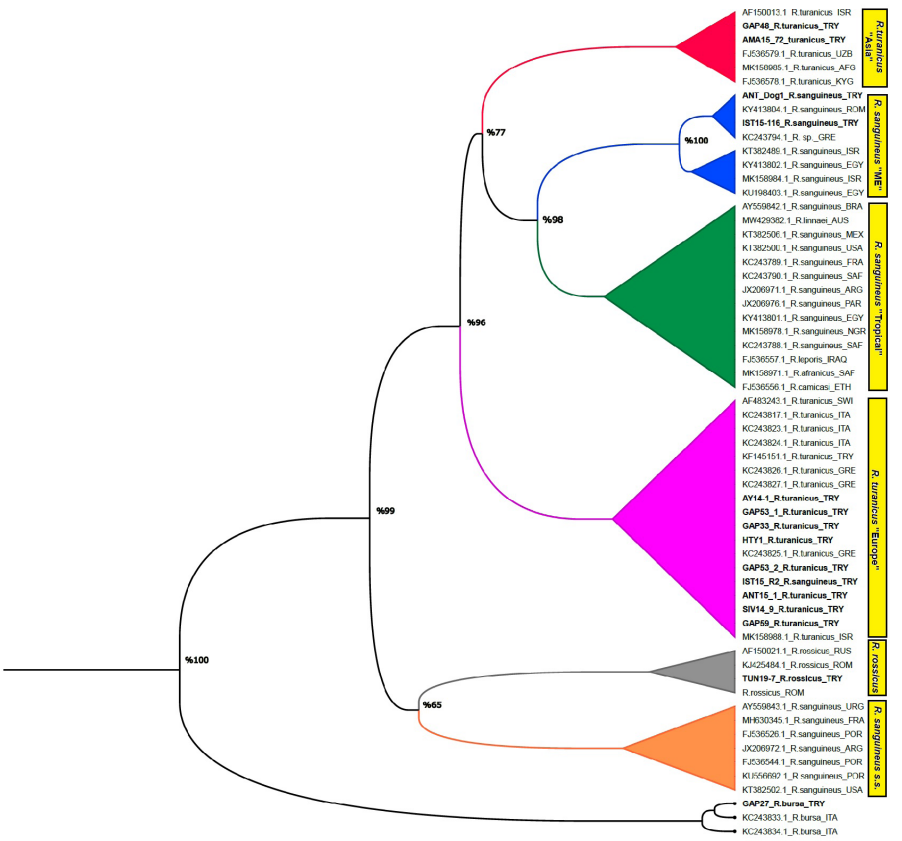

Figure 3. Phylogenetic tree of sequences obtained by mt 12S rDNA from this study and sequences of GenBank. Haplotypes obtained from this study are indicated with TRY codes and highlighted in bold.

distinguished. Samples belonging to the Middle Eastern lineage of *R. sanguineus* (CYP7, CYP9, ANT-Dog1), formed a distinct lineage (96%) like mitochondrial trees, but their clustering with *R. turanicus* samples makes their identification difficult. The reasons for this observation in the ITS2 marker are discussed in the discussion section.

3.4. Historical biogeography analysis

The S-DIVA and BBM models constructed using the mt 16S rDNA dataset provided different results for certain nodes (Figure 5). According to the S-DIVA analysis, the ancestor of the *R. sanguineus* complex exhibited a wide geographic distribution. This distribution range (Node 55:

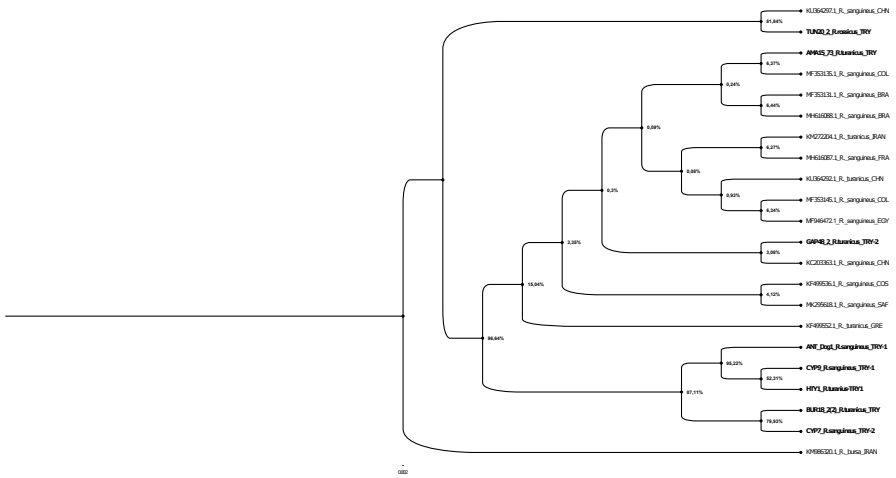

Figure 4. Phylogenetic tree of sequences obtained by ITS2 from this study and sequences of GenBank. Haplotypes obtained from this study are indicated with TRY codes and highlighted in bold. Different haplotypes of the same individual are labeled as TRY-1 and TRY-2 on the phylogenetic tree.

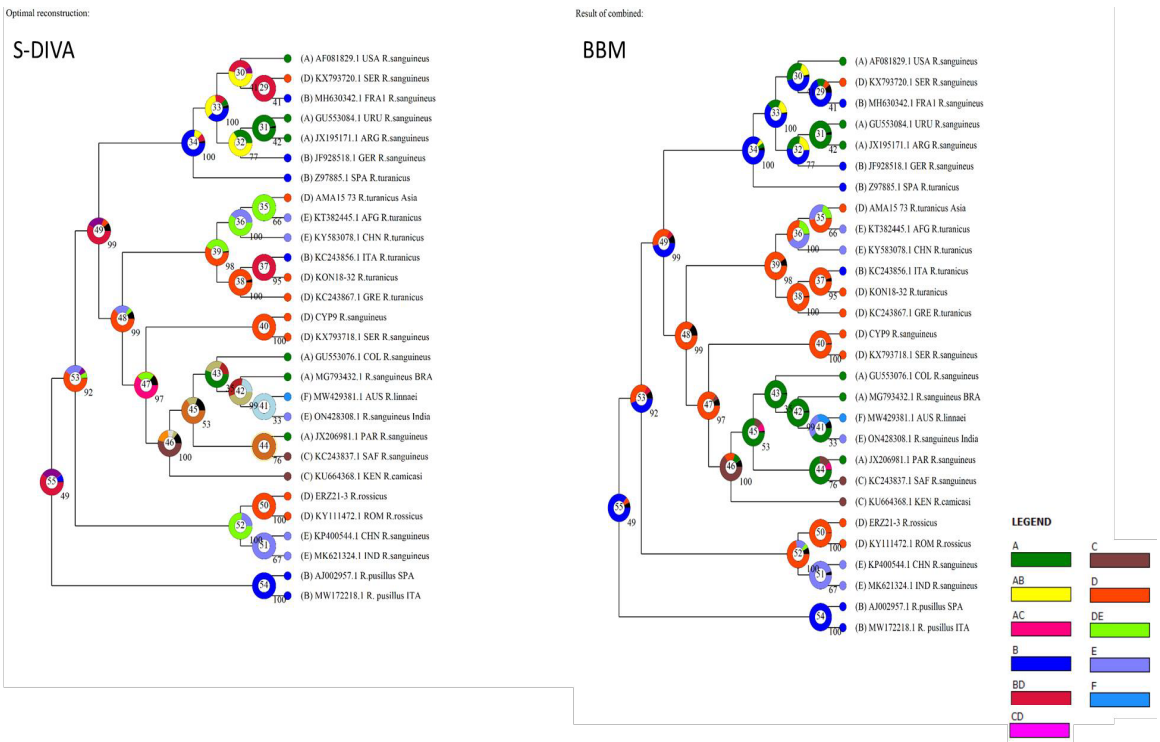

Figure 5. The biogeographic analysis of the *Rhipicephalus sanguineus* complex with S-DIVA and BBM analysis based on mt 16S rDNA.

B + D = 60.5%) encompassed the entire Europe and the Middle East. However, the BBM model identified Western Europe as the ancestor of the *R. sanguineus* complex (Node 55: B = 87.5%). Both models indicated that the ancestor of *R. pucillus* was from Western Europe (Node 54: B = 100%). The ancestor of lineages other than *R. pucillus* (Node 53) was either the Middle East + Eastern Europe according to the S-DIVA (D = 60%) or either Western Europe (B = 44.7%) or the Middle East + Eastern Europe (D = 38.7%) according to the BBM. The ancestor of *R. rossicus*, which was included for the first time in ancestral biogeography analyses, was determined as the Middle East + Eastern Europe by the BBM model (Node 52: D = 65%). According to S-DIVA, it was more extensive, including Asia as well (Node 52: D + E = 77%). The ancestor of taxa other than *R. rossicus* (Node 49) was Europe and the Middle East according to S-DIVA (B + D = 57.6%), while it was either Western Europe or Eastern Europe + the Middle East (B = 43.3%, D = 32.5%) according to the BBM. One of the lineages derived from this clade (Node 34) was *R. sanguineus* s.s., whose ancestor was Western Europe in both models (S-DIVA: B = 77%, BBM: B = 88.8%). The ancestor of Node 48, which is consisted of Asian and European lineages of *R. turanicus*, and *R. sanguineus* Middle Eastern + tropical lineage, was the Middle East + Eastern Europe according to both models (S-DIVA: D = 62%; BBM: D = 85%). Both models suggested that this common ancestor diverged into *R. sanguineus* tropical lineage in Africa and subsequently spread to America and Australia while also constituting *R. sanguineus* Middle Eastern lineage in the Middle East + Eastern Europe (Figure 5).

According to the mt 12S rDNA results of S-DIVA, the common ancestor of *R. sanguineus* complex exhibited ambiguity, and multiple alternatives existed (Figure 6). S-DIVA suggested a widely distributed ancestor encompassing Europe and the Middle East (B + D = 35%), the Middle East + Eastern Europe (D = 33%), or Europe, the Middle East, and Africa (B + C + D = 29%) origin. In contrast, BBM proposed Middle East + Eastern Europe (Node 55: D = 64.9%) origin. Although the ancestor of *R. rossicus*, *R. sanguineus* s.s., and *R. pucillus* (Node 54) was estimated as Europe and the Middle East (B + D = 100%) according to the S-DIVA, BBM indicated that the ancestor originated in the Middle East and Eastern Europe (D = 48.6%). Both models suggested that this ancestor split into two lineages: whereas *R. rossicus* originated in the Middle East + Eastern Europe (Node 48, S-DIVA: D + E = 100%; BBM: D = 51.7%), the origin of *R. sanguineus* s.s. and *R. pucillus* was America and Western Europe (Node 53, S-DIVA: A + B = 84%; BBM: B = 45%). BBM analysis postulated that the ancestor of the *R. sanguineus* tropical lineage and the *R. sanguineus* Middle Eastern lineage (Node 46) was the Middle East + Eastern Europe (D = 67%), while S-DIVA placed this taxon to be of Africa + the Middle East and Eastern Europe (C + D = 100%). Both models suggested that one branch diverged from this ancestor (Node 37) and remained in the Middle East + Eastern Europe (D = 100%), while the other lineage (Node 45) separated and migrated to Africa, then to America and Australia (C = 100%). S-DIVA estimated that ancestors of *R. turanicus* originated in Middle East + Eastern Europe or Middle East + Eastern Europe + Asia (Node 35: D = 46%; D + E = 54%). However, BBM placed the origin of *R. turanicus* in either Middle East + Eastern Europe or Asia (Node 35: D = 42%, E = 36%). This ancestor diverged into *R. turanicus* Asian and European lineages (Figure 6).

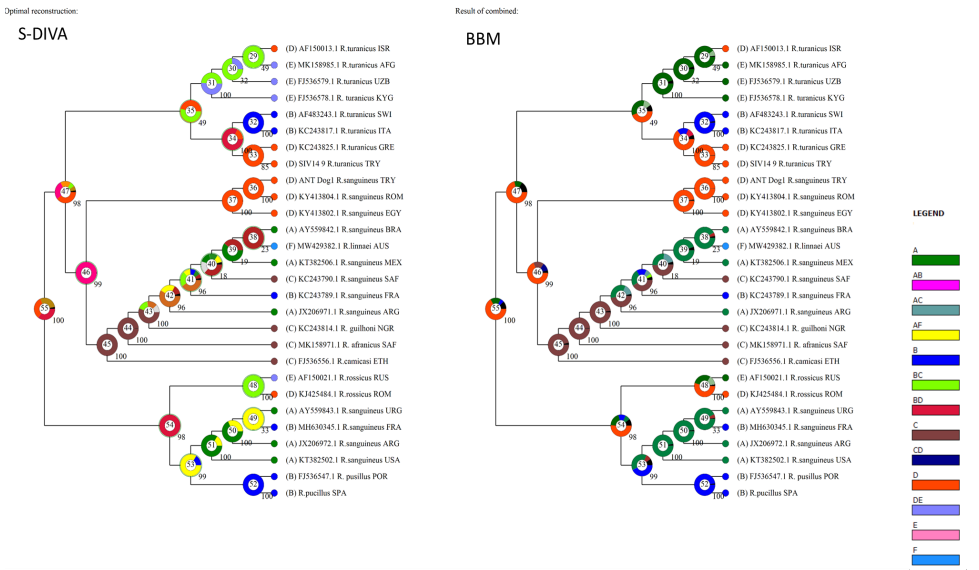

Figure 6. The biogeographic analysis of the *Rhipicephalus sanguineus* complex with S-DIVA and BBM analysis based on mt 12S rDN.

4. Discussion

4.1. Evaluation of the phylogenetic findings

This study has addressed the phylogeny of *R. sanguineus* complex comprehensively by combining the most commonly preferred gene regions in recent studies. The trees reconstructed from the mitochondrial markers were largely consistent with each other and with previous findings; however, some differences have been observed. For instance, mt 12S rDNA tree placed *R. rossicus* and *R. sanguineus* s.s. as closely related taxa (Figure 3). This can be explained by several factors such as different genes having different evolutionary histories, the length of sequences and the geographic region covered by the datasets. ITS2 has been the most preferred nuclear marker in studies on the phylogeny of *Rhipicephalus* species (Zahler et al., 1997; Murrell et al., 2001; Latrofa et al., 2013; Nava et al., 2018). However, the phylogenetic analyses conducted using this gene region have shown that ITS2 was incapable of distinguishing members of this complex (Zahler et al., 1997; Latrofa et al., 2013; Nava et al., 2018). One of the primary causes for this could be the approximately 300 bp length of the obtained sequences, which may not provide enough genetic information to distinguish the taxa. In contrast, mt 16S rDNA sequences, which cover all regions of the world and have sufficient length to reflect genetic differences between lineages, are commonly preferred in studies on the *R. sanguineus* complex and even other tick species.

The results of this study corroborated recent findings, including the presence of a genetically different Middle Eastern lineage within *R. sanguineus* tropical lineage and the separation of *R. turanicus* into Asian and European lineages (Bakkes et al., 2020; Hekimoglu et al., 2021). The detection of both the temperate and tropical lineages of *R. sanguineus* in Eastern Europe (Serbia, Croatia, and Romania) (Hornok et al., 2017) has raised the possibility that both taxa are also present in Türkiye, which is geographically close to these countries and shares similar biotic and abiotic conditions. The results of this study, however, showed that the tropical lineage is not present in Eastern Europe or Türkiye. Instead, the lineage distributed in these areas (Eastern Europe and Middle East) is the Middle Eastern lineage.

Although phylogenetic analyses indicated that some taxa are restricted to particular areas, *R. sanguineus* tropical lineage is considered to be the most successful taxon in terms of widening its range and colonizing to different continents. The distribution of this lineage in South America and Africa has been previously documented (Szabó et al., 2005; Moraes-Filho et al., 2011; Dantas-Torres et al., 2013; Latrofa et al., 2013). The species name has been changed to *R. linneai* after its finding in Australia (Slapeta et al., 2021). Our trees revealed the presence of this taxon in Western Europe (Figure 3, GenBank Accs number: KC243789) and North America (Figure 2, GenBank Accs number: KT382476 and Figure 3, GenBank Accs number: KT382500). More recently, it has been predicted that the species will continue to expand northward in North America (Pascoe et al., 2022). All these studies and the phylogenetic trees reconstructed in this study demonstrated that this species is distributed across all regions of the world except Eastern Europe + Middle East. To understand the reasons behind this, firstly, more extensive sampling (especially from dogs) should be conducted in these regions and to be totally sure that this species is not present here. Then, underlying biotic and abiotic factors should be investigated to clarify this.

Rhipicephalus rossicus, which was neglected in the majority of previous phylogenetic studies, has been extensively evaluated in this study by generating new sequences using different markers to understand its local and global distribution patterns and genetic relationship with other members of the complex. This species seemed to have a wide geographic distribution from Eastern Europe (Serbia, Romania, Croatia) to the Middle East (Türkiye) and then to the Asian continents (China, Russia, India) (Figure 2). In some parts of Asia and Middle East, it coexists with *R. turanicus* Asian lineage and *R. sanguineus* Middle Eastern lineage. This suggests the need for extensive research to determine whether the genetic differentiation between these species is the result of introgression or hybridization.

In light of recent phylogenetic findings from different regions of the world and systematic revisions on *R. sanguineus* complex, it has been necessary to update the data on the presence and distribution of the complex in Türkiye, which has great importance on the distribution of this complex to the different parts of the world (Hekimoglu et al, 2016). New locality records have been provided with this study. For instance, *R. rossicus* has only been documented in Tunceli Province (Hekimoglu et al., 2021); however, in this study, it was also recorded in neighboring Erzincan Province (Table 1). *Rhipicephalus turanicus* European lineage, which has been previously identified as the prevalent member of the *R. sanguineus* complex in Türkiye (Hekimoglu et al., 2016; Hekimoglu et al., 2021). The distribution of this taxon was demonstrated in Central, Thrace, Aegean (Hekimoglu et al., 2016), Eastern and Southeastern Anatolia (Hekimoglu et al., 2021), and demonstrated also with this study in the Mediterranean and Black Sea regions. These supplementary findings indicate that this lineage is present in almost all regions of Turkey. The presence of Asian lineage of *R. turanicus* in the Southeastern Anatolia has been reported (Hekimoglu et al., 2021). The existence of this lineage has been explained by transporting these ticks with livestock from

Asia to the Southeast Anatolia region of Türkiye, which may have favorable bioecological conditions for the establishment of populations of this taxon (Hekimoglu et al., 2021). The new record in Amasya Province showed that its transportation with hosts such as livestock or dogs continues towards the inner parts of the country, and the biotic and abiotic conditions in these regions may be also suitable for colonizing of this lineage in these areas (Figures 2 and 3). This study also clarified that *R. sanguineus* s.l. samples of Türkiye, which were previously designated as *R. sanguineus* tropical lineage, were in fact *R. sanguineus* Middle Eastern lineage. The presence of this taxon has been documented in the northern and western parts of Türkiye, but it has a broader geographical distribution comprising Aegean (Aydin), Mediterranean (Antalya) and Northern (İstanbul) regions. Samples from Northern Cyprus also grouped within this taxon (Table 1, Figure 2).

4.2. Evaluation of ancestral area analysis

The origin of *R. sanguineus* s.l. and its distribution from this ancestral area to different regions of the world has remained unclear for many years. In a previous study (Hekimoglu et al., 2016), two scenarios proposed different ancestors: S-DIVA indicated that *R. sanguineus* s.l. had a wide distribution encompassing Europe and the Middle East, which later diverged into two lineages. Subsequently, the Western European lineage colonized the Americas, while the Eastern European and Middle Eastern lineage colonized parts of Africa, Asia, and the Americas. BBM suggested a single European origin colonizing Western Europe, then Eastern Europe, the Middle East, Africa, and America (Hekimoglu et al., 2016). According to mt 16S rDNA, our analyses are consistent with the aforementioned study, whereas mt 12S rDNA suggested the Middle East + Eastern Europe as the origin of the *R. sanguineus* complex. The potential reasons for this difference may be associated with the properties of markers, which have been discussed in the previous section. On the other hand, both mitochondrial markers pointed out Middle East + Eastern Europe as the origin of several lineages of *R. sanguineus* complex such as *R. rossicus* and Asian and European lineages of *R. turanicus*. These findings once again highlighted the significance of the Middle East, Eastern Europe, and Türkiye in the global distribution of the complex's members.

One of the hypotheses tested in this study is whether the tropical and temperate lineages of *R. sanguineus* from Eastern Europe have diverged into distinct species and encountered in this region later or whether they have always coexisted there. Firstly, this hypothesis needed to be revised since our phylogenetic trees demonstrated that *R. sanguineus* tropical lineage did not exist in Eastern Europe. Instead of *R. sanguineus* tropical lineage, *R. sanguineus* Middle Eastern lineage has been replaced. Ancestral analysis demonstrated that *R. sanguineus* Middle Eastern lineage originated from the Middle East + Eastern Europe (Figures 5 and 6), indicating that this lineage never left its origin. Considering that the origin of *R. sanguineus* s.s. is Western Europe, this lineage appeared to have migrated to Eastern Europe, but not to the Middle East or Türkiye. Thus, it was suggested that both lineages of *R. sanguineus* have long been present in Eastern Europe.

In this study, several species and taxa were included in ancestral analyses for the first time. For instance, *R. rossicus* was previously reported in Romania (Mihalca et al., 2015; Dumitrache et al., 2014; Sandor et al., 2014), Croatia (Hornok et al., 2017), Eastern Siberia (Khasnatinov et al., 2016), and Türkiye (Hekimoglu et al., 2021). Additionally, this study demonstrated that GenBank samples from China and India, that were identified as *R. sanguineus*, were in fact *R. rossicus* (Figure 2). Our analyses revealed that the ancestor of *R. rossicus* originated from the Middle East and Eastern Europe before migrating to Asia (Figures 5 and 6). Another taxon included in the analysis for the first time was the Asian lineage of *R. turanicus*. Likewise, this lineage originated Eastern Europe + Middle East and later colonized to Asia. *Rhipicephalus sanguineus* Middle Eastern lineage also originated from the Middle East + Eastern Europe, with one lineage remaining in this region and the other expanding to Africa, America, and Australia (Figure 6). The question of whether these three lineages populate to areas where they are not currently found is crucial for future research. Additionally, gene flow and introgression between populations should be examined to gain a more comprehensive understanding of the genetic relationships between these taxa.

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Supplementary Table 1. Locality and reference information of sequences downloaded from GenBank used in phylogenetic trees.

No	Genbank accession number	Locality	Reference	Gene region
1	AF081829.1	USA	Black and Roehrdanz, 2016	16S
2	JX304708.1	France	René-Martellet et al., 2017	16S
3	KX793720.1	Serbia	Hornok et al., 2017	16S
4	KX793724.1	Croatia	Hornok et al., 2017	16S
5	MH630342.1	France	Nava et al., 2018	16S
6	GU553081.1	Spain	Moraes-Filho et al., 2011	16S
7	JX195171.1	Argentina	Nava et al., 2012	16S
8	JF928518.1	Germany	Hoffman et al., 2012	16S
9	GU553084.1	Uruguay	Moraes-Filho et al., 2011	16S
10	MH630343.1	France	Nava et al., 2018	16S
11	Z97885.1	Spain	Mangold et al., 1997	16S
12	KF219733.1	Israel	Erster et al. 2013, unpublished	16S
13	MZ463291.1	Türkiye	Hekimoglu et al., 2021	16S
14	MZ463292.1	Türkiye	Hekimoglu et al., 2021	16S
15	KC203362.1	China	Lv et al., 2014	16S
16	KT382459.1	Kyrgyzstan	Zemtsova et al., 2016	16S
17	KY583074.1	China	Li et al., 2017	16S
18	MF002559.1	China	Guo et al., 2017, unpublished	16S
19	KY583078.1	China	Li et al., 2017	16S
20	KF219734.1	Israel	Erster et al. 2013, unpublished	16S
21	KF219736.1	Israel	Erster et al. 2013, unpublished	16S
22	KP866203.1	Eastern Siberia	Khasnatinov et al., 2016	16S
23	KT382445.1	Afghanistan	Zemtsova et al., 2016	16S
24	KF219730.1	Israel	Erster et al. 2013, unpublished	16S
25	KF219731.1	Israel	Erster et al. 2013, unpublished	16S
26	KX793728.1	Croatia	Hornok et al., 2017	16S
27	KC243856.1	Italy	Dantas Torres et al., 2013	16S
28	KX793723.1	Croatia	Hornok et al., 2017	16S
29	KC243867.1	Greece	Dantas Torres et al., 2013	16S
30	KX793721.1	Montenegro	Hornok et al., 2017	16S
31	MZ463294.1	Türkiye	Hekimoglu et al., 2021	16S
32	KU664358.1	Türkiye	Hekimoglu et al., 2016	16S
33	KU664367.1	Türkiye	Hekimoglu et al., 2016	16S
34	MZ463286.1	Türkiye	Hekimoglu et al., 2021	16S
35	KY945492.1	Egypt	Senbill et al., 2017	16S
36	KU664365.1	Türkiye	Hekimoglu et al., 2016	16S
37	KX793718.1	Serbia	Hornok et al., 2017	16S
38	KR870984.1	Türkiye	Orkun et al., 2018	16S
39	KY945493.1	Egypt	Senbill et al., 2017	16S
40	KY413783.1	Egypt	Chitimia-Dobler et al., 2017	16S
41	KY413785.1	Egypt	Chitimia-Dobler et al., 2017	16S

42	KY413790.1	Romania	Chitimia-Dobler et al., 2017	16S
43	GU553076.1	Colombia	Moraes-Filho et al., 2011	16S
44	MK680295.1	Meksika	Lopez-Perez et al., 2019	16S
45	KC243838.1	Colombia	Dantas Torres et al., 2013	16S
46	JX206981.1	Paraguay	Nava et al., 2012	16S
47	KC243837.1	S. Africa	Dantas Torres et al., 2013	16S
48	KT382476.1	USA	Zemtsova et al., 2016	16S
49	MG793432.1	Brazil	Ramos, 2018	16S
50	MG793435.1	Brazil	Ramos, 2018	16S
51	ON428308.1	India	Bhowmik et al., 2022	16S
52	MW429381.1	Australia	Slapeta et al., 2021	16S
53	MW429382.1	Fiji	Slapeta et al., 2021	16S
54	KU664368.1	Kenya	Hekimoğlu et al., 2016	16S
55	KU664368	Kenya	Hekimoğlu et al., 2016	16S
56	MZ463289.1	Türkiye	Hekimoğlu et al., 2021	16S
57	KP866202.1	Russia	Khasnatinov et al., 2016	16S
58	MZ463288.1	Türkiye	Hekimoğlu et al., 2021	16S
59	KY111472.1	Romania	Langguth et al., 2017	16S
60	KP400544.1	China	Du et al., 2015	16S
61	MK621323.1	India	Senbill et al., 2017	16S
62	MK621324.1	India	Senbill et al., 2017	16S
63	KP400542.1	China	Zhang et al., 2015	16S
64	KU664348.1	Türkiye	Hekimoğlu et al., 2016	16S
65	KU664349.1	Türkiye	Hekimoğlu et al., 2016	16S
66	KU664350.1	Türkiye	Hekimoğlu et al., 2016	16S
1	AF150013.1	Israel	Beati and Keirans, 2001	12S
2	FJ536579.1	Uzbekistan	Santos Silva and Beati, 2009, unpublished	12S
3	MK158985.1	Afghanistan	Bakkes et al., 2020	12S
4	FJ536578.1	Kyrgyzstan	Santos Silva and Beati, 2009, unpublished	12S
5	KY413804.1	Romania	Chitimia-Dobler et al., 2017	12S
6	KC243794.1	Greece	Dantas Torres et al., 2013	12S
7	KT382489.1	Israel	Zemtsova et al., 2016	12S
8	KY413802.1	Egypt	Chitimia-Dobler et al., 2017	12S
9	MK158984.1	Israel	Bakkes et al., 2020	12S
10	KU198403.1	Egypt	Abdullah et al., 2015, unpublished	12S
11	AY559842.1	Brazil	Szabo et al., 2005	12S
12	MW429382.1	Australia	Slapeta et al., 2021	12S
13	KT382506.1	Mexico	Zemtsova et al., 2016	12S
14	KT382500.1	USA	Zemtsova et al., 2016	12S
15	KC243789.1	France	Dantas Torres et al., 2013	12S
16	KC243790.1	S. Africa	Dantas Torres et al., 2013	12S
17	JX206971.1	Argentina	Nava et al., 2012	12S
18	JX206976.1	Paraguay	Nava et al., 2012	12S
19	KY413801.1	Egypt	Chitimia-Dobler et al., 2017	12S

20	MK158978.1	Nigeria	Bakkes et al., 2020	12S
21	KC243788.1	S. Africa	Dantas Torres et al., 2013	12S
22	FJ536557.1	Ethiopia	Santos Silva and Beati, 2009, unpublished	12S
23	MK158971.1	S. Africa	Bakkes et al., 2020	12S
24	FJ536556.1	Iraq	Santos Silva and Beati, 2009, unpublished	12S
25	AF483243.1	Switzerland	Bernasconi et al., 2002	12S
26	KC243817.1	Italy	Dantas Torres et al., 2013	12S
27	KC243823.1	Italy	Dantas Torres et al., 2013	12S
28	KC243824.1	Italy	Dantas Torres et al., 2013	12S
29	KF145151.1	Türkiye	Dantas Torres et al., 2013	12S
30	KC243826.1	Greece	Dantas Torres et al., 2013	12S
31	KC243827.1	Greece	Dantas Torres et al., 2013	12S
32	KC243825.1	Greece	Dantas Torres et al., 2013	12S
33	MK158988.1	Israel	Bakkes et al., 2020	12S
34	AF150021.1	Russia	Beati and Keirans, 2001	12S
35	KJ425484.1	Romania	Dumitrache et al., 2012	12S
36	AY559843.1	Uruguay	Szabo et al., 2005	12S
37	MH630345.1	France	Nava et al., 2018	12S
38	FJ536526.1	Portugal	Santos Silva and Beati, 2009, unpublished	12S
39	JX206972.1	Argentina	Nava et al., 2012	12S
40	FJ536544.1	Portugal	Santos Silva and Beati, 2009, unpublished	12S
41	KU556692.1	Portugal	Almeida et al., 2017	12S
42	KT382502.1	USA	Zemtsova et al., 2016	12S
43	KC243833.1	Italy	Dantas Torres et al., 2013	12S
44	KC243834.1	Italy	Dantas Torres et al., 2013	12S
1	KU364297.1	China	Wang et al., 2017, unpublished	ITS2
2	MF353135.1	Colombia	Rivera-Paez et al., 2017	ITS2
3	MF353131.1	Brazil	Rivera-Paez et al., 2017	ITS2
4	MH616088.1	Brazil	Nava et al., 2018	ITS2
5	KM272204.1	Iran	Nabian et al., 2014, unpublished	ITS2
6	MH616087.1	France	Nava et al., 2018	ITS2
7	KU364292.1	China	Wang et al., 2017, unpublished	ITS2
8	MF353145.1	Colombia	Rivera-Paez et al., 2017	ITS2
9	MF946472.1	Egypt	Senbill et al., 2017	ITS2
10	KC203363.1	China	Lv et al., 2014	ITS2
11	KF499536.1	Costa Rica	Latrofa et al., 2013	ITS2
12	MK295618.1	S. Africa	Van wyk et al., 2019	ITS2
13	KF499552.1	Greece	Latrofa et al., 2013	ITS2
14	KM986320.1	Iran	Amiri et al., 2015, unpublished	ITS2